

Reflections

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From Proteins and Protein Models to Their Use in Immunology and Immunotherapy

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The two leitmotifs of my scientific research were synthetic polymeric models of proteins and the structure of proteins as reflected by the three-dimensional conformation controlled by their amino acid sequence. This in turn led me to synthetic polypeptide antigens and to investigation of antibodies, ultimately resulting in the discovery of the determinant-specific genetic control of immune response and to the development of a therapeutic vaccine against the exacerbating-remitting form of multiple sclerosis. In protein chemistry I had two great teachers who became very close friends, Ephraim Katchalski-Katzir and Christian B. Anfinsen (Fig. 1), but in immunology I lacked the benefit of a great mentor, and so to some extent I was self-taught. Thus, I had to “figure out” many things myself.

My Attraction to Polymers

In my childhood I grew up in the courtyard of a textile factory, and I later intended to study fibers, mainly the new synthetic ones. However, I first started studying macromolecules when, as a Ph.D. student, in 1950 I joined the laboratory of Ephraim Katchalski at the Weizmann Institute of Science. Ephraim first synthesized polylysine in the early 1940s and was successfully exploring the use of polyamino acids as protein models. With his collaborators he prepared and studied many physical, chemical, and biological properties of several polymers of trifunctional amino acids such as polyarginine, polyaspartic acid, polyhistidine, and polyserine, as well as the polyamino acids polyproline and polyhydroxyproline (1, 2). Among the early syntheses was the one of poly-L-tyrosine, which was part of the subject of my Ph.D. thesis. My thesis research included also the synthesis of poly-3,5-diiodotyrosine and poly-*p*-amino-L-phenylalanine. Later on I was directly involved with the synthesis of polytryptophan and polycyclohexylalanine. Of special interest was the spectrophotometric titration of polytyrosine and of copolymers of tyrosine with positively or negatively charged amino acids; this showed the influence of the vicinal electrostatic field on the ease of ionization of the phenolic hydroxyl groups (2, 3). We were very touched when John Edsall included figures from this study in his book *Biophysical Chemistry* with Jeffreys Wyman. Actually *Proteins, Peptides and Amino Acids as Dipolar Ions* (Cohn and Edsall's book) served as a basis for intense seminars with both of the Katchalski-Katzir brothers, Ephraim and Aharon. The accent was on physicochemical properties of proteins, polyamino acids, and polyelectrolytes in general. Ephraim was a remarkable teacher—stimulating, inspiring, patient, and always friendly. Today I still cherish his friendship.

For the preparation of poly-L-tyrosine, I remember that I tried 40 times to synthesize the monomer, *N*-carboxy-*O*-benzyloxycarbonyl-L-tyrosine anhydride, before I was successful. This taught me the need for perseverance and optimism in research. Of course, serendipity (defined as luck meeting the prepared mind) also helps. I mention this because much of the story that follows actually depended on the availability of this monomer (4).



FIG. 1. Christian B. Anfinsen, Ephraim Katzir-Katchalski, and Michael Sela (1986).

My first paper was on the titration of *N*-carboxy- α -amino acid anhydrides in nonaqueous solvents (5). These are the monomers in the synthesis of amino acid polymers, and the titration was useful for the determination of unreacted anhydride at any instant. My Ph.D. thesis was on the azo derivatives of some aromatic poly- α -amino acids (6). One could even diazotize poly-*p*-aminophenylalanine and couple it to polymers containing tyrosine, resulting in colored water-insoluble compounds.

I shall describe later how the work with polyamino acids brought me into immunology. What I would like to mention here is that we could use as initiators of polymerization of *N*-carboxyamino acid anhydrides (the monomers from which polyamino acids were built) not only monofunctional small molecules but also macromolecules possessing several amino groups. If proteins were used as such polyvalent initiators, we ended up with polypeptidyl proteins, whereas when polylysine or polyornithine were used, we had for the first time multichain polyamino acids, which became so important for me later as synthetic antigens. Mark Stahmann also prepared such polypeptidyl proteins in Wisconsin. We summarized in *Advances in Protein Chemistry* in 1958 (1) and 1959 (2) the early studies on the synthesis and the chemical and biological properties of poly- α -amino acids.

In 1955 I was back in Europe as a young scientist presenting the work on multichain polyamino acids at the International Congress of Chemistry in Zurich, and a week later, the work on spectrophotometric titration of polymers and copolymers of amino acids at the International Congress of Biochemistry in Bruxelles. This was a wonderful occasion to meet scientists whom until then I had known only by reputation and through their papers. Among the many I want to mention two, Hans Neurath and Bill Harrington, who later became close friends of mine. During the same trip I went to see Sir Charles Harington in London. Harington was the man who elucidated the structure of thyroxine and predicted that it is formed from two diiodotyrosine residues by oxidation rather than simple dismutation. I wanted to tell him that the availability of polytyrosine had permitted us (7) to find out that, following alkaline incubation of iodinated polytyrosine, the hydrolysate contained 2% thyroxine and 2% serine. We had, thus, proved the validity of his prediction.

Later I shall discuss our work on linear and multichain polyamino acids as synthetic polypeptide antigens, but now I want to mention that ultimately we developed a therapeutic drug/vaccine against the exacerbating-remitting stage of multiple sclerosis, denoted copolymer 1, named glatiramer acetate and Copaxone by the industry. This drug, approved by the Food and Drug Administration in 1996 and used by tens of thousands of patients, is the first polymeric drug/vaccine in which the active ingredient is the polymer itself, in contrast to polymers used earlier in pharmacopeia for slow release of drugs or for wrapping them.

As a result of studying the mechanism of polymerization of *N*-carboxyamino acid anhydrides with Arie Berger, I was involved in an interesting story that I want to relate here while discussing polyamino acids. On my second visit to the National Institutes of Health (NIH) in 1960, Marshall Nirenberg came one day and asked me whether I had some poly-L-phenylalanine and whether I knew its solubility properties. I did not have the polymer in Bethesda, but I did ask Nirenberg why he was interested. Through these conversations I became one of the first to know about the breaking of the genetic code, UUU encoding Phe. Although I was somewhat skeptical of the story, I immediately looked for and found, hidden somewhere in an experimental section of a paper in the *Journal of the American Chemical Society*, that poly-L-phenylalanine was insoluble in all the solvents we had tested with the exception of a saturated solution of anhydrous hydrogen bromide in glacial acetic acid (8). Because on that very day I was preparing just such a solution (used to remove carbobenzoxy groups) in the laboratory, I gladly gave the reagent to Nirenberg and was touched and surprised when he acknowledged this in the classical paper that resulted in his receiving the Nobel Prize. The real point of the story lies elsewhere. Why did we try to use such a peculiar solvent? The truth of the matter is that years earlier, together with the late Arie Berger in Rehovot, we were investigating the mechanism of polymerization leading to linear and multichain polyamino acids. One day I had two test tubes, one with polyphenylalanine and one with polycarbobenzoxyllysine, stuck in a beaker on my desk. Arie came to decarbobenzoxylate the lysine polymer, a reaction with hydrogen bromide in glacial acetic acid during which carbon dioxide is released. He took the wrong test tube away with him and returned, puzzled because the material had dissolved and he could not see any evolution of carbon dioxide. At once we realized the mistake and I noted in my laboratory book that, at long last, we had found a solvent for poly-L-phenylalanine.

Ribonuclease and Other Enzymes

After 5 years of working with protein models, I felt ready to go abroad for a postdoctoral period to work with proteins. Thus, I arrived in 1956 in the laboratory of Chris Anfinsen at the NIH in Bethesda. The friendship between us resulted in prolonged stays in Bethesda in 1956–1957, 1960–1961, and 1973–1974. On his part, Chris came to Rehovot on sabbaticals on several occasions and was an active, extremely valuable member of the Board of Governors of the Weizmann Institute of Science, serving for many years as the Chairman of its Scientific Advisory Committee. His death in May 1995 was for me personally a deeply felt loss. Chris was a great friend, helpful to all those who surrounded him, full of charm and modesty, and actually a great romantic.

From the NIH we sent out our first joint paper, which Chris was in a hurry to prepare for a Festschrift honoring Linderstrom-Lang. The topic was the selective splitting of protein chains by trypsin at arginine residues after lysine residues were reversibly blocked by carbobenzoxy groups. Oxidative opening of the four disulfide bridges of bovine pancreatic ribonuclease to permit its sequencing was possible only because tryptophan was absent in this protein. A more general method, which could be used also for a protein containing tryptophan, such as lysozyme, was reductive cleavage, followed by blocking of the sulfhydryl groups with iodoacetic acid (9). I was fortunate to participate with Chris in these studies and left part of the reduced ribonuclease without blocking its sulfhydryl groups to see if it could reoxidize properly and whether the enzymatic activity would come back. The results of these experiments (10) showed a total recovery of the activity even though statistical considerations pointed to 105 various ways in which the four disulfide bridges could reform. Thus, we demonstrated that no additional information was needed for the correct, unique architecture of a protein molecule and that it is the genetic code dictating the amino acid sequence that is responsible for the conversion of the randomly coiled structure. It is for this most important observation that in 1972 Chris was awarded the Nobel Prize in chemistry, which he shared with Stanford Moore and William Stein, who established the amino acid sequence of ribonuclease. I learned a lot from Chris, a dedicated scientist, who had an incredible flair for attacking the right protein and the most elegant experiment to solve it, a flair matched only by his literary talent.

During my visits to Bethesda, I tried in two ways to combine my previous experience with polyamino acids and research on enzymes. One was the use of copolymers of glutamic acid with aromatic amino acids to efficiently inhibit ribonuclease (11), lysozyme, or trypsin. The other series of studies had to do with poly-DL-alanylation of proteins. In contrast to poly-L-alanine

and poly-D-alanine, poly-DL-alanine, which is a random copolymer of L-alanine and D-alanine, is well soluble in water and may serve as a solubilizer. Poly-DL-alanyl ribonuclease could be reduced and reoxidized properly. Poly-DL-alanylation renders gliadin water-soluble (12) and converts myosin into a derivative soluble in distilled water and with all its ATPase activity preserved (13).

On a lighter level, when I arrived in Bethesda everybody was writing the sequences of amino acids in straight lines; the disulfide bridges (at straight angles to the chain) occupied as much space as 10 residues. I found a huge hole puncher with which I made round pieces of paper, wrote the name of 1 residue on each piece of paper, and played with them until the half-cystines of a bridge were able to touch each other. This gave rise to the well known "swan" shape of ribonuclease, and it delights me to see that proteins are still often schematically presented like this.

After returning from my first visit to the NIH (1956–1957), I embarked on a collaboration with Nathan Citri on two conformationally different states of penicillinase. The enzyme changes from an iodine-resistant to an iodine-sensitive state in urea or guanidine hydrochloride. The change in conformation was followed by optical rotatory measurements. This, in 1960, was my first of many papers published in the *Journal of Biological Chemistry* (14).

During my second stay at the NIH (1960–1961), I continued studies on oxidation of reduced ribonuclease (15) and started investigating the enzymatic properties of poly-DL-alanyl derivatives of ribonuclease (16, 17), trypsin, and chymotrypsin (18). Alanylated ribonuclease with as many as 4 or 5 alanine residues per chain kept its enzymatic activity and regenerated its full activity after reduction of its disulfide bridges and subsequent reoxidation (16). We later learned that alanylation affected the enzymatic activity toward RNA but not toward the low molecular weight substrates (17). Poly-DL-alanyl trypsin, with an average polyalanine chain length of 6–9 residues, was resistant to autolysis at temperatures up to 38 °C and reacted normally with soybean and serum inhibitors. Poly-DL-alanyl chymotrypsin had similar stability and activity properties (18). Several years later, Roger Acher visited us from Paris and investigated poly-DL-alanyl Kunitz trypsin inhibitor (19). Only when the alanine residues were attached exclusively to the α -amino group was the inhibitor active. Upon alanylation of all the amino groups, the derivative was virtually inactive, demonstrating the crucial role of ϵ -amino groups for its activity.

While still in Bethesda, I checked the inhibition of ribonuclease by copolymers of glutamic acid and aromatic amino acids (11). Copolymers of tyrosine (or phenylalanine) and glutamic acid are much more efficient inhibitors of ribonuclease than polyaspartic acid, and in this case the attachment to the enzyme occurs not only through electrostatic interactions but also through short range urea-labile bonds. The digestion of ribonucleic acid by pancreatic ribonuclease was stopped completely, at pH 5.0, by relatively small amounts of the inhibitory copolymer. We tested these copolymers also for inhibition of lysozyme (20) and trypsin (21) with similar results.

Immunogenicity and Antigenic Specificity

As my Ph.D. thesis was concerned with polytyrosine (a polymeric chain of phenols) and poly-*p*-aminophenylalanine (a polymeric chain of anilines), it was natural to produce polypeptidic azo dyes from them. I reasoned that these might serve as synthetic models for azoproteins, of which one rare example, provided by Landsteiner, was the attachment of haptens including peptides, via an azo bond, to proteins. Reading Landsteiner's book *The Specificity of Serological Reactions*, I came across the statement that gelatin probably is not antigenic because it contains no tyrosine. This led me to study the possibility of increasing the antigenicity of gelatin by attachment of tyrosine peptides. To do these studies, the amino groups of the protein were used to initiate the polymerization of the tyrosine monomer, as mentioned earlier. The continuation of this study was the Ph.D. thesis of Ruth Arnon, and we showed that limited tyrosylation enhanced immunogenicity without significantly changing specificity, whereas more extensive tyrosylation converted gelatin into a potent immunogen provoking antibodies mainly to tyrosyl peptides (22). It was at this time that we first promoted the notion of immunogen and immunogenicity and distinguished it from antigenic specificity.

I was delighted to describe the results on the increase in immunogenicity of gelatin upon its polytyrosylation to Sir Charles Harington on the same visit in 1954, as it was in his laboratory that John Humphrey some 15 years earlier had tried to increase the antigenicity of gelatin by

attaching it chemically to carbobenzoxy-L-tyrosine. It was several years later that we started collaborating with John Humphrey and his colleagues on several aspects of synthetic polypeptide antigens, contributing to a better understanding of immunology.

As a result of the studies on tyrosylated gelatin, we assumed that gelatin was not necessary for immunogenicity. We, therefore, replaced the gelatin with multichain poly-DL-alanine (23, 24) as the carrier for peptides of tyrosine and glutamic acid and showed that the resulting copolymer, denoted (T,G)-A-L, led to specific, precipitable antibodies in experimental animals. At that stage Sara Fuchs joined us, and we synthesized numerous linear and multichain polyamino acids and tested them for immunogenicity. Our preliminary communication on a "synthetic antigen" was rejected by *Nature* under the pretext that the journal does not publish papers that are part of a series, so we published it elsewhere (25). The final paper (26) became a Citation Classic (*Current Contents*, 1986). The availability of synthetic antigens permitted a systematic elucidation of the molecular basis of antigenicity (27–29). We could learn a lot about the role of size, composition, and shape as well as about the accessibility of those parts of the molecule crucial for immunogenicity. As a matter of fact, we learned that it was possible (provided one was prepared to invest the necessary effort) to prepare synthetic immunogens leading to antibodies of essentially any specificity.

Although in most cases a good immunogen had a molecular mass of at least several thousand daltons, dinitrophenyl-hexalysine and arsanil-trityrosine were by themselves capable of triggering an efficient immune response. The minimal size for a molecule to be immunogenic depends, therefore, largely on its chemical nature.

Although electrical charge may be important in defining the antigenic specificity of an epitope, charge is not a minimum necessary cause for immunogenicity; we could prepare water-soluble amino acid copolymers devoid of charge that were immunogenic. Polymers of D-amino acids were immunogenic only when they were administered in minute amounts and they led to no secondary response. Their immunogenicity was thymus-independent, as was that of several other polymers such as linear (Pro-Gly-Pro)_n and multichain polymers of L-proline locked in with terminal polymeric side chains of D-Phe and D-Glu. The common denominator of all these "thymus-independent" antigens was that they not only possessed repeating antigenic determinants but they also were metabolized slowly, if at all. Even though polymers composed exclusively of D-amino acids are "thymus independent," we were able to show more recently that they are capable of inducing the formation of T cell hybridomas. The different roles of D-amino acids had intrigued me for many years, and I summarized the topic recently (30). Most of the above studies on the molecular basis of antigenicity were carried out before the crucial role of T lymphocytes in immune response was realized. Only later the central question of immunology became: T-B or not T-B?

In the early days there was a wonderful feeling working on synthetic antigens because practically nobody else was working on the subject, but later on it was as pleasant and satisfying to know that so many laboratories had become interested in the synthetic approach to immunological phenomena. One of the most fascinating aspects of our studies with synthetic antigens had to do with the steric conformation of the immunogen and of its epitopes. We distinguished between conformational (conformation-dependent) and sequential determinants (31) and showed how the same peptide (Tyr-Ala-Glu) may lead to antibodies recognizing the sequence (when attached to multichain poly-DL-alanine) or recognizing an epitope defined by conformation (when the tripeptide was polymerized to give an α -helical structure). In addition, we could demonstrate for the first time, by circular dichroism, how antibodies to the α -helical polymer could help transconform into a helical shape a small polymer that was not yet helical (32). These studies led us directly to study proteins and to synthesize a macromolecule in which a synthetic "loop" peptide derived from hen egg white lysozyme was attached to branched polyalanine (33). The resulting antibodies reacted with intact lysozyme through the "loop" region, but the reaction was totally abolished when the disulfide bond within the "loop" was opened, and thus the three-dimensional structure was collapsed. In this connection, it should be remembered that partial degradation products of proteins may still be immunogenic. Furthermore, the sera we were investigating might have contained a myriad of antibodies against degradation products derived from the original immunogen.

In later years we showed that a peptide of 20 amino acid residues derived from the coat protein of MS2 bacteriophage induced, after conjugation with an appropriate carrier, the formation of antibodies that cross-reacted with the intact virus (34). More recently, tens of

peptides analogous to segments of proteins have been prepared that may lead to antibodies cross-reactive with the intact protein. Nevertheless, one should remember that many similar peptides have been prepared that were not capable of provoking anti-protein responses. The extent of cross-reactivity will depend entirely on the probability that the free peptide will be able to attain the conformation that it possesses in the native protein. This capability may be prevented either when the segment is too short, not yet able to possess a stabilized correct conformation, or when it is too long and possesses a preferred stabilized conformation different from the one capable of cross-reaction. If the protein segment is more flexible, the chance of cross-reaction is higher, even though there will be cases when a small peptide is capable of manifesting a relatively rigid conformation similar to the one it possesses within a native protein. Thus, antibodies to the rigid helix (Pro-Gly-Pro)_n cross-reacted with collagen (35). The exposure to the outside (hydrophilicity) is also very important to immunopotency: "Whatever sticks out most, is most immunopotent."

Synthetic antigens allowed the study of antibody specificity, immunological tolerance, the role of net electrical charge in defining the nature of antibodies, and delayed hypersensitivity. The results led us to the inevitable conclusion that an immunogen is much more than an antigenic determinant attached to an inert carrier. Unfortunately, we did not know that the separate recognition of antigen by T and B cells was the explanation for our results. However, we did clearly state that the "carrier" had a crucial role in defining the nature of the immune response toward an epitope. Similar to the cooperation between T and B cells for antibody formation, delayed hypersensitivity might be the result, we suggested, of cooperation between two distinct sets of T cells. Recently, I came back to this problem when reviewing together with Israel Pecht, "The Nature of Antigen" (36).

Antibodies

Being interested in methods for isolating antibodies we purified antibodies to gelatin from antigen-antibody complex by proteolysis (37). In another case (38), we isolated on columns of Sephadex antibodies to such low molecular weight antigens as lysozyme and a synthetic polypeptide. With Ruth Arnon we succeeded in preparing an analog of the Fab dimer by splitting the IgG molecule with cyanogen bromide rather than with proteolytic enzymes (39, 40).

Poly-DL-alanylation of immunoglobulin because of its great solubilization effect permitted the total reduction of all the disulfide bridges within the molecule without insolubilizing the light and heavy chain products. This allowed for controlled reoxidation, leading to the correct association of light and heavy chains and the recovery of both antigenic and antibody activity (41, 42). It also confirmed the hypothesis that no genetic information other than that present in the amino acid sequence of the polypeptide chains is required for the correct conformation of a protein molecule as complex as immunoglobulin G.

When Israel Pecht returned to Rehovot from Manfred Eigen's laboratory, he extended the interest in the antibody-combining site to its kinetic aspects. Using temperature-jump methodologies, Pecht was able to resolve the hapten-recognition process with the first homogeneous antibody available at that time, namely a dinitrophenyl specific IgA myeloma (43).

Of great interest to me was the observation we made with Edna Mozes on the inverse electrical net charge relationship between an antigen and the antibodies it provoked (44, 45). The more positively charged the antigen, the more negatively charged was the antibody. We showed that this depended on the net charge of the intact antigen, not on local clustering of charges around the epitope. Thus, we proved that the epitope is recognized while the antigen is still intact. The phenomenon holds for different classes of antibodies and is also valid at the cellular level but not for thymus-independent antigens.

In those early days we used antibodies attached to bromoacetylcellulose for the purification of antigens, and we used antigens (similarly insolubilized) for the isolation of antibodies (46, 47). We did not call it as yet "affinity chromatography," but these were among the first examples of this approach to purification. A detailed study of antibody-combining sites to a series of peptide determinants of increasing size and defined structure led us to the conclusion that the size of the combining sites was in all cases such as to accommodate four amino acid residues and that the most exposed portion of the epitope plays an immunodominant role (48, 49). In two interesting studies, we could show how the combining site of the antibody can transconform the structure of the antigen. I mentioned earlier how antibodies to the α -helical

polymer could help transconform a small polymer that was not yet helical into a helical shape (32). We could also demonstrate that the Fab of an antibody to *p*-azobenzenearsonate hapten may “suck out” the *p*-azobenzenearsonate moiety from its unavailable conformation within an ordered copolymer and convert it into another conformation, recognized by Fab (50).

Genetic Control of Immune Response

Even though some hints could be found in earlier literature, the actual establishment of the genetic control of the immune response became possible only through the study of synthetic antigens, simple chemically, in inbred strains of mice and guinea pigs, simple genetically.

In our studies (51, 52), we first showed determinant-specific (antigen-specific) genetic control of immune responses by making use of multichain polyamino acids as antigens and inbred mice as experimental animals. The multichain synthetic polypeptides we investigated, possessed small amounts of tyrosine, histidine, or phenylalanine at the tips of their polymeric side chains. These antigens were denoted (T,G)-A-L, (H,G)-A-L, and (Phe,G)-A-L. We noted that when histidine was substituted for tyrosine, genetic control was completely reversed, whereas replacement with phenylalanine led to a material strongly immunogenic in both strains investigated.

Some time later, using these multichain polypeptides, Hugh McDevitt was able to show for the first time the link between the immune response and the major histocompatibility locus of the mouse, which in turn led to our present day understanding of immune response genes and their products. Of all the contributions of synthetic polypeptides toward our present day understanding of immunology, none has been more important than the discovery and the definition of the genetic control of the immune response, which in turn was a crucial trigger toward a better understanding of the cellular basis of immunological responsiveness. Not surprisingly, a very large proportion of studies using synthetic polypeptides in immunology has been devoted to this field of research. As is apparent from the above story, my contribution has been mainly chemical and immunochemical, whereas McDevitt contributed the major part of the genetic aspects of this study. He described it in detail in his recent scientific autobiography (53).

Synthetic Vaccines

I mentioned earlier, while discussing the role of steric conformation in defining antigenic specificity, that we had prepared a totally synthetic antigen capable of provoking antibodies reacting with native lysozyme. These studies led to the inevitable conclusion that a new approach to vaccination was possible. We reasoned that synthetic vaccines might be a reality in the future (54) for the simple reason that if these conclusions held for one protein, they may hold for others, including viral coat proteins and bacterial toxins. Of course, it is not sufficient to have just a synthetic epitope that will provoke antibodies to the protein. I shall not repeat here the arguments I have made before as to why there is a place for improvement of vaccines today, but for a synthetic vaccine to be successful, it should contain at least five ingredients: (a) specificity; (b) built-in adjuvanticity; (c) the correct genetic background; (d) the capacity to cope with antigenic competition; and (e) the correct “texture,” *i.e.* a form that will give persistent and long lasting immune protection. Much of the experimental work was done in collaboration with Ruth Arnon and various other colleagues.

We first synthesized a peptide from the amino terminus of the carcinoembryonic antigen of the colon; this showed a weak cross-reaction with the intact antigen (55). The first study related to viruses was the synthesis of a peptide from the envelope of the MS2 bacteriophage (34). The synthetic peptide inhibited phage neutralization by antiphage antibodies, and the same peptide elicited antibodies capable of neutralizing the virus after attachment to multichain poly-DL-alanine. Similarly, Ruth Arnon succeeded in preparing a conjugate of a synthetic peptide derived from influenza hemagglutinin, and it provoked antibodies and protected mice against influenza challenge. With Chaim Jacob, we showed that tetanus toxoid coupled with synthetic peptides of the B subunit of cholera toxin led to the formation of antibodies capable of neutralizing the toxic activity of the native cholera toxin (56). Actually, some antibodies inhibited the entire spectrum of activities of the intact cholera toxin, including adenylate cyclase induction and intestinal fluid secretion. Attachment of a peptide composed of residues 50–64 within the sequence of the B subunit of cholera toxin to our multichain polymer (T,G)-A-L produced a totally synthetic vaccine, which elicited in rabbits antibodies with neutralizing capacity (57).

In the above studies, we used Freund's adjuvant or water-soluble peptidoglycan as an adjuvant. A short while later, in collaboration with Louis Chedid and Francoise Audibert, we used their synthetic muramyl dipeptides to prepare totally synthetic conjugates in which a synthetic antigenic determinant and a synthetic adjuvant were covalently linked to a synthetic carrier (58). The resulting conjugate, when administered in aqueous solution into experimental animals, provoked the formation of protective antibodies. When the muramyl dipeptide was bound covalently, it was much more efficient than when it was first mixed with the antigen. We prepared, with Ruth Arnon, such totally synthetic antigens, and these led to neutralization of a virus, MS2 (59), as well as to protection against diphtheria and cholera (60).

Copolymer 1 and Multiple Sclerosis

In our early studies with Ruth Arnon, of special interest was the immune response to lipid components, which was not easy to either elicit or investigate because of solubility problems. However, conjugates in which synthetic lipid compounds were attached onto synthetic copolymers of amino acids elicited a specific response to lipids such as cytolipin H, which is a tumor-associated glycolipid (61), or sphingomyelin (62). Furthermore, we demonstrated that both the sugar and lipid components of such molecules contributed to their immunological specificity. The resultant anti-lipid antibodies were capable of detecting the corresponding lipids both in water-soluble systems and in their physiological milieu. This was fascinating because it gave us a glimpse into some disorders involving lipid-containing tissue and consequently led to our interest in demyelinating diseases, namely disorders in which the myelin sheath, which constitutes the lipid-rich coating of all axons, is damaged, resulting in various neurological dysfunctions. We thus thought that EAE (experimental allergic encephalomyelitis) caused by MBP (myelin basic protein) might actually be induced by a demyelinating lipid and that the positively charged MBP might serve only as a schlepper (carrier) for an acidic lipid (*e.g.* phospholipid). We prepared several positively charged copolymers of amino acids and tested whether we could induce EAE when the copolymers were administered into experimental animals (guinea pigs and rabbits) in complete Freund's adjuvant, similarly to the successful administration of MBP, but we failed. On the other hand, the injection of several positively charged amino acid copolymers in aqueous solution into mice, rabbits, and guinea pigs resulted in efficient suppression of the onset of the disease, experimental allergic encephalomyelitis (63–65). Later on, we could suppress the actual disease in rhesus monkeys and baboons (65, 66). The copolymer 1 we primarily used, denoted Cop 1, now called glatiramer acetate, and by industry "Copaxone," is composed of a small amount of glutamic acid, a much larger amount of lysine, some tyrosine, and a major share of alanine. To our pleasant surprise, there is a significant immunological cross-reaction (both at the antibody level (67, 68) and at the T cell level (69, 70)) between Cop 1 and the myelin basic protein. Interestingly, when an analog of Cop 1 made from D-amino acids was tested, it had no suppressing capacity nor did it cross-react immunologically with the basic protein (71). Cop 1 is not generally immunosuppressive; it is not toxic; actually it is not helpful in any other autoimmune disease except in multiple sclerosis and its animal model, experimental allergic encephalomyelitis.

The clinical trials with Cop 1 have included two preliminary open trials and two double-blind phase II trials, one involving exacerbating-relapsing patients (72) and another one in chronic progressive patients (73). The results of the phase II trial in exacerbating-relapsing patients demonstrated a remarkable decrease in the number of relapses and in the rate of progression in Cop 1-treated patients compared with the placebo control. Cop 1 is a promising, low risk multiple sclerosis-specific drug for treatment of the relapsing disease. As an antigen-specific intervention, Cop 1 has the advantage of reduced probability of long term damage to the immune system.

After a successful, pivotal multicenter phase III clinical trial conducted in 11 medical centers in the United States (74), Cop 1 was approved by the United States Food and Drug Administration as a drug for multiple sclerosis. This was a moment of gratification and deep emotion for my colleagues and myself as well as for our industrial partners, Teva Pharmaceutical Industries.

We were obviously very interested in the mode of action of Cop 1. We know that the effect was specific for the disease, and we assumed that it has to do with the immunological cross-reaction between the "villain" (myelin basic protein) and the drug (Cop 1). What we have learned later is that Cop 1 binds almost immediately and strongly to the groove of major

histocompatibility complex (MHC) class II antigens of most genetic backgrounds, and it displaces efficiently from the groove any peptides derived from the myelin basic protein (75). This promiscuity is probably because of its polymeric character permitting microheterogeneity in the amino acid sequence. The extensive and promiscuous binding to class II MHC molecules, without prior processing, leads to clustering of these molecules on the antigen-presenting cells, which may explain their high affinity binding (76).

This is the first necessary but not sufficient step in its mechanism of action. The binding, which is the least specific step, is a prerequisite for its later effects. Following this interaction, two mechanisms were clearly shown to be effective. 1) Cop 1 binding to the relevant MHC leads to the activation of T suppressor cells because of suppressive determinants shared between myelin basic protein and Cop 1. 2) Successful competition between the complex of Cop 1-MHC class II antigen with the complex of myelin basic protein-MHC class II antigen for the myelin basic protein-specific T cell receptor (a phenomenon called by immunologists the “T receptor antagonism”) is shown (77).

An important step in our understanding of the mode of action of Cop 1 was the observation that copolymer 1 induces T cells of the T helper type 2 that cross-react with myelin basic protein and suppress experimental autoimmune encephalomyelitis (78). This was corroborated by clinical studies in multiple sclerosis patients (79). It was of interest to observe that Th2 suppressor lines and clones induced by Copolymer 1 cross-reacted at the level of Th2 cytokine secretion with myelin basic protein but not with other myelin antigens (80). This bystander suppression may explain the therapeutic effect of Cop 1 in EAE and multiple sclerosis (MS).

Cop 1 binds promiscuously to many different cells regardless of their DR restriction. It binds avidly and fast and can also displace already bound antigens, and this holds for all the myelin antigens that may be involved in MS; and yet, Cop 1 exerts its activity in an antigen-specific manner (it is not a general immunosuppressive agent and does not affect other experimental autoimmune diseases). Its specificity must, therefore, be envisaged in the context of the trimolecular complex MHC-Ag-T-cell receptor (“the immunological synapse”), namely as interference with the presentation of the encephalitogenic antigen to the T-cell receptor, which is a specific interaction.

I summarized recently the story of specific vaccines against autoimmune diseases (81) as well as the successful use of Cop 1 (glatiramer acetate, Copaxone) in the treatment of multiple sclerosis for exacerbating-relmitting patients (82). The majority of the patients in the great clinical trial continue to be followed in an organized fashion for more than 7 years. Their risk of an MS relapse was over 1.5 per year at onset and is now less than 1 every 6 years. On average, these patients have experienced no increase in neurological disability, whereas natural history profiles would have predicted substantial worsening. The accumulated experience with glatiramer acetate (Cop 1) indicates that its efficiency is apparently increased as a function of usage time while the favorable side effect profile is sustained.

Personally, the whole odyssey of Cop 1 and its use in MS has been a source of great satisfaction and emotion. The awareness that tens of thousands of MS patients feel better because of a drug/vaccine that we conceived and developed moves me deeply. Twenty-eight years passed from the moment of the idea to the approval of Cop 1 by the Food and Drug Administration. I have a feeling that discoveries resulting from basic research take a longer time to fruition, but on the other hand, they are probably more original in terms of concept.

I shall not describe here recent results on the use of Cop 1 to inhibit the progression of secondary degeneration after crush injury of the rat optic nerve (83). Cop 1 also offered protection from retinal ganglion cell loss resulting from a direct biochemical insult caused by glutamate and in a rat model of ocular hypertension (84). This study may point the way to a therapy for glaucoma, a neurodegenerative disease of the optic nerve often associated with increased intraocular pressure as well as for acute and chronic degenerative diseases in which glutamate is a prominent participant.

Therapeutic Vaccines and Autoimmunity

Vaccines are prophylactic in the sense that they are administered to healthy individuals to prevent a disease. Nevertheless, there is a growing trend to use vaccines to alleviate the suffering of those already having a disease. Great effort is being devoted to develop vaccines against tumors, AIDS, hepatitis, tuberculosis, Alzheimer’s disease, Huntington disease, etc.

Copolymer 1, used today as a vaccine against MS, is a good example of a beneficial treatment for this autoimmune disease based on its similarity to MBP, one of the putative causes of MS (82). This finding could lead to therapeutic vaccines against other autoimmune diseases such as myasthenia gravis (MG), juvenile diabetes, systemic lupus erythematosus, and rheumatoid arthritis. Furthermore, antibodies prepared against prions raise hopes for a vaccine against bovine spongiform encephalitis and Creutzfeldt-Jakob disease, and antibodies to a peptide derived from β -amyloid plaques could degrade plaques and be used as a therapeutic vaccine against Alzheimer's disease.

By its definition, a preventive vaccine is sufficiently similar in its chemistry to the molecule that provokes the disease so that the immune response directed against it can act against the causative agent. This situation is analogous in the case of therapeutic vaccines. At least one "therapeutic vaccine," copolymer 1 (glatiramer acetate) for the relapsing-remitting form of multiple sclerosis, is being used by many thousands of patients. Another vaccine for type I diabetes has recently completed a phase II trial successfully, and several vaccines against cancer are already being studied, some of which are planned to enter (or have just begun) clinical trials. Therapeutic vaccine preparations against infectious diseases such as HIV, tuberculosis, and malaria are in phase II clinical trials to evaluate their efficacy in patients. In most cases the therapy is based on the resemblance between the etiological agent causing the disease and the therapeutic vaccine.

What is characteristic for a vaccine is its specificity. You do not have one vaccine against all kinds of different viruses or bacteria. For every troublemaker, there is a "molecular cousin," close enough in its chemical composition to lead to an immune response cross-reactive with the troublemaker but harmless biologically because the danger of the original virus or bacterial toxin has been knocked out.

Myasthenia Gravis

Multiple sclerosis is mainly a T cell-mediated disease, whereas in myasthenia gravis the attack of specific antibodies on the acetylcholine receptor (AChR) is the accepted cause of disease. Nevertheless, assuming that most antibody responses need helper T cells, we have synthesized two immunodominant myasthenogenic T cell epitopes (p195–212 and p259–271) derived from an α -subunit of the nicotinic AChR (85). Ideally, the goal of therapy for MG should be the elimination of autoimmune responses to the AChR specifically, without interfering with immune responses to other antigens. To this end, the dual analog composed of the tandemly, reciprocally arranged two single analogs of p195–212 and p259–271, namely Lys-262–Ala-207, was prepared and shown to efficiently inhibit the proliferation of T cell lines specific to the myasthenogenic peptides and of lymph node cells primed *in vivo* to either of these peptides. The dual analog specifically inhibited *in vitro* T cell stimulation to either myasthenogenic peptide in >90% of the responding MG patients (86). The dual analog interferes with specific autoimmune responses (87), and when administered orally, the dual analog could treat experimental allergic myasthenia gravis (EAMG) induced in mice by immunization with the multideterminant native *Torpedo* AChR (88). Moreover, it had beneficial effects on the clinical manifestations characterizing EAMG. Thus, the dual analog is an efficient immunomodulator of EAMG in mice and could be of specific therapeutic potential for MG.

Cancer Research

The idea of binding anti-cancer therapeutic drugs covalently to antibodies reacting with cancerous cells has appealed to me from an early time. Instead of having the drugs, given systemically, spread throughout the whole body, immunotargeting would focus the supply of the drug exclusively to the cancer area. However, we did not get to immunotargeting until many years later when we bound daunomycin and adriamycin via a dextran bridge to antibodies against antigens of leukemia, lymphoma, and plasmacytoma cells. We showed that these are effective as "guided missiles" both *in vitro* and *in vivo* (89). The Fab dimers were almost as effective as intact antibodies. Daunomycin linked to anti-tumor antibodies penetrated the cell membrane at a higher rate than daunomycin linked to dextran or to normal immunoglobulin (90). Together with Japanese colleagues we could show a chemotherapeutic effect against hepatoma in rat (91).

With Meir Wilchek we showed indirect immunotargeting of cis-platinum to a human epidermoid carcinoma using the avidin-biotin system (92). The biotinylated antibody was

attached to the cancer cell, and this was followed by cis-platinum attached to avidin. We moved to monoclonal antibody against the extracellular domain of the epidermal growth factor receptor, denoted today ErbB1, and found that its conjugate with daunomycin was quite efficient but so was the antibody by itself (93). A strong synergistic effect was observed when the anti-ErbB1 antibodies were administered together with cis-platin. This observation became of great interest because of its therapeutic potential (e.g. in the review article by Mendelsohn and Baselga (94)). Over the years I became more and more convinced that what matters most is the nature of monoclonal antibodies.

In the last dozen years I have been collaborating with Yossi Yarden, who has been working on the family of ErbB receptors and their ligands. We produced and investigated antibodies against these interesting protooncogene products. ErbB2 (also known as HER-2/Neu) is a tyrosine kinase, and its dense appearance is correlated with a poor prognosis in breast cancer. The antibodies formed either inhibited or accelerated the tumorigenic growth of ErbB2-transfected fibroblasts in athymic mice (95). Suppression and promotion of tumor growth by monoclonal antibodies to ErbB2 differentially correlated with cellular uptake (96). ErbB2 has no known efficient ligand but has tyrosine kinase activity. On the other hand, ErbB3 has a ligand, heregulin, but has no tyrosine kinase activity. When they heterodimerize, the dimer is an efficient and active receptor. ErbB2 heterodimerizes also with ErbB1 (epidermal growth factor receptor) and with ErbB4. Based on this information, we investigated (97) a large battery of monoclonal antibodies to ErbB2 and could divide them into several subclasses according to their biological activity. The inhibitory effect of one subclass was due to the acceleration of ligand dissociation by the blocking of heterodimerization. These observations may help us to understand the molecular mechanisms involved in the potential therapeutic effect of anti-ErbB2 antibodies.

Concluding Remarks

I have always been driven by curiosity and search. I know that there are individuals who prefer to be alone all the time, and maybe through daydreaming they reach all their working hypotheses, but I am a great believer in the interaction and in the fertilization of ideas. I have been collaborating with many colleagues around the world, and I have always been keen on having many visiting scientists spending extended periods of time in my laboratory. As for theories, they are very good as working hypotheses as long as you do not take them too seriously, but they are dangerous when they become dogmatic. Since I became involved with research, I have always had two precepts. If something is not worth doing at all, it is not worth doing very well; and if something is worth doing but is obvious, why should I do it? Only if I thought it is not obvious, I would embark on it.

I published my first paper in the *Journal of Biological Chemistry* in 1960 and the last one in 2000, a span of 40 years, including 17 publications.

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