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Review

Fiftieth anniversary of the description of the chemical structure of antibodies

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ABSTRACT

The identification of the antitoxic property of serum in 1890 by Emil von Behring and the introduction of the term "Antikörper" by Paul Ehrlich in 1891 referring to one of the most relevant mechanisms of defense of the adaptive immune system, ie, the humoral immune response mediators, mark the beginning of modern immunology. The "Y" structure was described 50 years ago by Gerald M. Edelman and Rodney R. Porter. Thus, on the fiftieth anniversary of the description of the chemical structure of antibodies, we consider it appropriate to celebrate this fact by sketching a brief outline and review of these epoch-making achievements..

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Cincuentenario del descubrimiento de la estructura química de los anticuerpos

RESUMEN

La identificación de la propiedad antitóxica del suero en 1890 por Emil von Behring y la introducción del término "Antikörper" por Paul Ehrlich en 1891 para referirse a uno de los mecanismos de defensa más relevantes del sistema inmunitario adaptativo, es decir, los mediadores de la respuesta inmunitaria humoral, marcan el inicio de la etapa de la inmunología moderna. La estructura en "Y" fue descrita hace cincuenta años por Gerald M. Edelman y Rodney R. Porter. Así, al cumplirse el cincuentenario de la descripción de la estructura química de los anticuerpos, consideramos oportuno no dejar pasar inadvertido el hecho a través de una breve remembranza y la revisión de dichos hallazgos.

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Science has created its own heraldry, a group of insignias that exemplify its achievements, its field, its fashion, and its future. In the biology of our time, the 2 most elegant and stylish insignia are the double helix of DNA and the Y of the immunoglobulin molecule. The Y is one of the best-characterized molecules, and an extensive book could be written about it. But we will only concern ourselves on reviewing the road and the obstacles that had to be faced in order to elucidate its chemical structure, 50 years ago.

Background

We could place the start of acquired immunity's history in the year 400 BC when Thucydides, in his book *The Peloponnesian War*, when referring to the plague that affected Athens noted that: " those who had survived the disease showed more compassion toward the dead and the sick, because they knew it well and now felt safe. Because no one suffered it a second time, at least not to die from it."¹

For the next 2300 years it was considered that as a consequence of a disease, the body became immune, because it lost an essential nutrient for that certain miasma, precisely because the malignant effluvium had consumed it its first—and not necessarily mortal—visit. This concept was capable of satisfactorily explaining the origin, specificity and variability in the duration of the immunity for different disease. But immunology, during this time lapse, was not yet a science.

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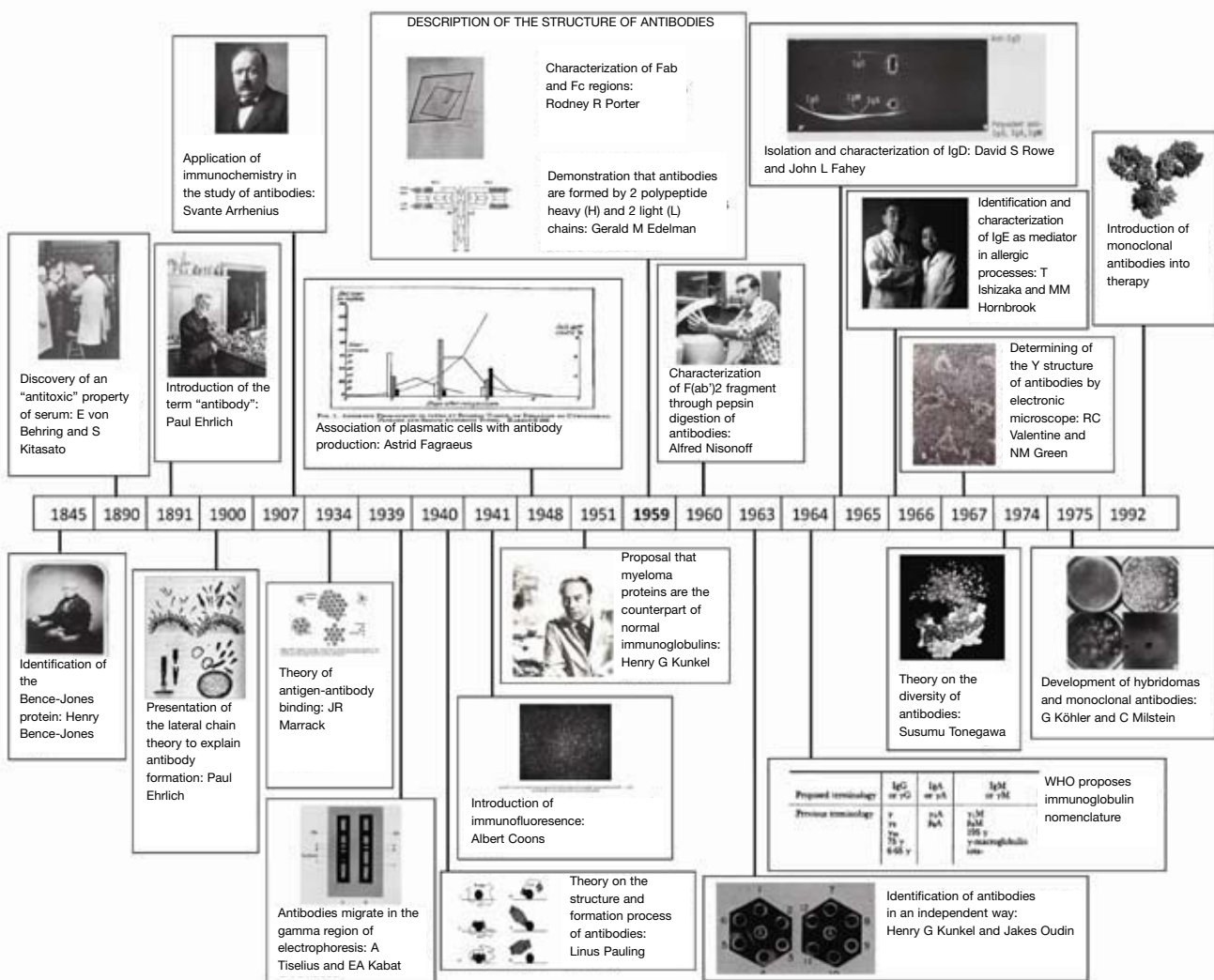


Figure 1. Principal contributions in characterizing and identifying antibodies throughout history. The figure shows the most important occurrences in the history of antibodies. There are, undoubtedly, omissions and not all aspects shown are treated in the text.

In 1890 Emil Behring and Shibasaburo Kitasato discovered serotherapy using the sera of rabbits immunized against tetanus and against diphtheria (Figure 1), and with that discovered that immunity is the acquisition of something—that may be transferred—, and not the loss of something that can undoubtedly not be transferred to a living organism.² One year later, on Christmas eve, the first application of serotherapy to a child with diphtheria was carried out, This led Behring to receive a knighthood (the von preceding his family name) and the first Nobel prize in Physiology or Medicine in 1901, unjustly not given also to Kitasato.

The experiment by Kitasato and Behring is unique in its type due to 2 reasons: a) it showed that the resistance to microbial disease can occur through sera, and b) it demonstrated passive immunity: the acquisition of resistance to pathogens through the transference of that property from an immunized donor. This experiment also opened the door to modern immunology, providing a concrete study subject that, although with an unknown chemical structure, at least to be expectant in the bloodstream, in sera. It must be mentioned that Kitasato and Behring only spoke of a specific antitoxic property in serum. Two Italians studying the tetanus toxin, Guido Tizzoni and Guiseppina Cattani, had discovered a substance (a salt-precipitating globulin) in 1891 and had named it antitoxin.³ That same year, Paul

Ehrlich reached the conclusion that when 2 different toxins (rycin and abrin) are administered to experimental animals, 2 different antikörper are generated, therefore introducing the term (antibody) that is still in use today.⁴

Immunochemistry

Although the chemist Louis Pasteur founded immunology, the father of immunochemistry was undoubtedly the physician Paul Ehrlich. In 1897 he pointed out the need to standardize antitoxin quantifications.⁵ His elaborate model (that included the hypothetical existence of toxoids) was based in his conception of singular atomic groups (functional-structural) in a toxin molecule. One of the groups was toxic; the adjacent one presented a complementary configuration to the host cell. The venomous portion could denaturalize to form an innocuous toxoid while at the same time maintaining its capacity to bind to the cell or an antibody. The variation in the avidity of the antigen-antibody reaction, therefore, was a question of complementarity of the molecular borders and clearly exemplified that fundamental approach by Ehrlich in all of his research: *corpora non agunt nisi fixata*, substances do not react unless fixed.

In 1900 he presented his surprising lateral chain theory before the Royal Society in London, in which antibodies are preformed membrane protein structures that, by being selected (not induced) by the antigen, increase their synthesis and the excess goes into the circulation. Although it was the first theory on antibody formation, it had a lot of critics among his contemporaries.⁶ All in all, Ehrlich obtained the Nobel Prize in Physiology or Medicine in 1908 due to his multiple contributions to immunology.

Although immunology was still in its infancy, it was one of the first biologic disciplines that obtained solid support in chemistry. In 1907, Svante Arrhenius (Nobel Prize in Chemistry 1903) came up with the term immunochemistry to refer to the union between chemistry and biomedical immunology, a science that would spend 50 years obsessed with the antigen-antibody reaction.⁷ Arrhenius was not completely in agreement with the emphasis set by Ehrlich in the molecular borders and, in contrast, postulated that antigens and antibodies were combined through a form of electrostatic colloidal binding. He believed that the union between antigens and antibodies shared similarities with the interaction between weak acids and bases. This physicochemical pioneer, whose experiments were very famous in his time—in spite being fundamentally flawed—, introduced into immunochemistry aspects of thermodynamics, equilibrium constants, viscosity coefficients, and other quantitative parameters that not only bound, but literally fused chemistry with immunology.

The first experimental evidence supporting the atomic group model proposed by Ehrlich was provided by Karl Landsteiner (Nobel Prize in Physiology or Medicine in 1930). He meticulously measured the specificity of antigen-antibody reactions to synthetic compounds with variations so subtle that they cleared any doubts on their exquisite specificity and would lead to his seminal textbook, still valid in spite of the passage of time.⁸ Landsteiner discovered the function of haptens and the carrier effect.⁹ With his experiments he gave the idea of antigenicity a material counterpoint, the specificity of the antibodies themselves.

At that time, the idea prevailed that only proteins, and maybe some glucoproteins, were the only chemical structures capable of stimulating the production of antibodies, because this never happened when animals were immunized with carbohydrates. However, in 1923, Michael Heidelberger and Oswald T. Avery, while studying the pneumococcal polysaccharide, discovered evidence to the contrary.¹⁰ Initially, the capsule polysaccharide of pneumococcus was recognized as an hapten, capable of binding to an antibody but incapable of inducing the production of it by itself. Afterwards, with the right dose and favorable hosts, it was demonstrated that polysaccharides were antigenic and, even more, immunogenic. Heidelberger, who was an organic chemist by profession, used the pneumococcus and other complex carbohydrates as tools for the quantitative microanalysis of immunologic specificity that, as he stated: “liberated the antigen-antibody reaction from the tyranny of titles.”¹¹

But immunology, which all in all had been considered a medical discipline in the first quarter of the 20th century, with clear therapeutic aspirations, by the end of the decade of the 1920s into a research gold mine for chemists. Colloidal chemistry was at the zenith of popularity although notions of polar and hydrophilic binding were being postulated. The atomic model of Niels Bohr was of capital importance for this, with its image of layers of electrons, leading to the concept of electron-valence.

During the next decade, in 1934, the British physician John R. Marrack proposed that hydrophilic forces (hydrogen binding) were the cause of antigen-antibody interactions. In addition, he described that if the antigens and some antibodies in particular could have a valence greater than the unit, several immunologic enigmas could be satisfactorily resolved such as flocculation, precipitation, and solubility of antigen-antibody complexes in antigen or antibody

excess zones. His hypothesis, based on Bohr's model of the atom, fought the wounded, but still prevalent, colloidal theory and the general consensus that antibodies were monovalent.¹²

The following years were filled with technological advances that allowed immunochemistry some certainty on the physical nature of the antibody, and all of this happened in Scandinavia. Between both world wars, Theodor Svedberg, in Sweden, stamped creativity on chemical technology. He obtained the Nobel Prize for Chemistry in 1926 and is mainly known for inventing the ultracentrifuge.¹³ In fact, sedimentation coefficients are measured in Svedberg (S) units in his honor. Between 1925 and 1932 Svedberg had Arne Tiselius as a student and later as an intimate collaborator, who himself obtained the Nobel Prize for Chemistry in 1948 for the discovery of another analytic method: electrophoresis. One destiny led to another hence, a disciple of Heidelberger, Elvin Kabat, performed post-doctoral studies with Tiselius in Uppsala, Sweden. This relationship had as a result the discovery of new and extraordinary techniques for the physical separation of antibodies from all other serum proteins, such as isoelectrofocusing and 2-dimensional immunoelectrophoresis. Tiselius and Kabat performed electrophoresis on the sera of egg albumin immunized rabbits and demonstrated that the activity of the antibody was found on the third spike of electrophoretic migration of proteins, also known as the gamma spike, which very soon led antibodies to be named gammaglobulins.¹⁴ When certain proteins from the gamma spike were determined not to be antibodies, the term immunoglobulins was introduced, generating the term gamma immunoglobulin (IgG). The analysis by ultracentrifuge established that gammaglobulins had a sedimentation coefficient of 7S, with a molecular weight close to 150 000 Da. However, not all antibodies are in this category. Those that migrated faster to the beta spikes were initially called γ M β 2-macroglobulins, known now as macro immunoglobulins (IgM). These have a sedimentation coefficient of 19S and an approximate molecular weight of 900 000 Da. Changes in the electrophoretic pattern of the serum was also seen during an immunization period. Therefore, formation of IgM began upon first encountering antigen, diminishing after a few days, as IgG increased. The subsequent challenges with antigen typically produced the same IgM response, but a greater quantity of IgG immediately appeared, leading to the term booster shot.

A finding of particular importance in the research by Tiselius and Kabat was that antibodies are not uniform in their electrical charge or their sedimentation coefficients, constituting the first evidence of the physical heterogeneity of antibodies.

In 1950 Tiselius received who would become the father of modern clinical immunology, Dr Henry Kunkel, who came from the Rockefeller Institute for Medical Research (today called Rockefeller University), and who came to Uppsala to perform a brief research fellowship. Kunkel rapidly assimilated all of the methodology of Tiselius' laboratory and dominated it. When he returned to New York, he had an idea of how to solve the analysis of antibody structure. As has been mentioned, their study was very complex due to their heterogeneity, making the performance of analytical studies with homogeneous molecules practically impossible. In 1951 he made a discovery of enormous reach. At this time, patients with multiple myeloma were thought to secrete products derived from malignant cells. In a series of experiments of stunning simplicity, he demonstrated that the elevation of proteins in the multiple myeloma patients' sera was related to normal gammaglobulins.¹⁵ This finding gave immunochemists the possibility to study homogeneous molecules to analyze and compare and made it ultimately possible to identify antibody classes, immunoglobulin chains, their genes, and constant and variable regions.

At the end of the decade, Kunkel made a classic contribution to rheumatology by demonstrating the presence of immune complexes in rheumatoid arthritis. He showed that rheumatoid factor is a

19°S IgM antibody directed against a 7°S IgG.¹⁶ He also discovered the presence of immune complexes with DNA and other cell components in the sera of patients with lupus erythematosus.¹⁷ The severity of disease could be related to the presence of circulating immune complexes. Both rheumatoid factor and the immune complexes were related, apparently, to the pathogenesis of arthritis and lupus. In addition, this served as a convincing example of the existence of autoantibodies at a time in which autoreactivity was far from being an established concept. Lastly, we must mention that the perception of Kunkel that myeloma proteins (monoclonal products of malignant plasma cells) were the equivalent of normal antibodies produced by plasma cells demonstrated that the theory of clonal selection postulated by Frank Macfarlane Burnet in 1959 was correct.¹⁸

Chemical structure of antibodies

When Rodney R. Porter (1917–1986) decided to perform molecular surgery on the antibody, he was unaware he was lighting the match that would start a fireworks show in immunology. Currently, the explosive rebirth of this science shows no signs of someday stopping. Porter studied at the Universities of Liverpool and Cambridge. Between 1949 and 1960 he worked at the National Institute for Medical Research, in Mill Hill. His last post was as Biochemistry professor at Trinity College in Oxford. When he started working in Mill Hill, biochemistry had flourished, especially in the field of methodology: compounds conjugated with radioisotopes, 2-dimensional paper chromatography and liquid column chromatography, both ion exchange as molecular size discrimination. In addition, it was known that proteolytic enzymes separated proteins by hydrolyzing their peptide unions, always in a precise place depending on the enzyme being used. Porter employed rabbit gammaglobulin and decided to chose papain (an enzyme that needs a reducing agent to activate it) to digest the structure, therefore isolating through liquid carboxymethylcellulose ion exchange chromatography, 2 fractions (I and II) apparently similar, and 1 fraction (fraction III) completely different.¹⁹ Although his work showed some muddled and confusing results, he managed to demonstrate that 2 of his fragments (I y II) presented affinity for binding antigen. Experimental data firmly indicated bivalency (the first 2 fragments were capable of binding antigen without precipitating, as predicted by monovalent structures), making Porter imagine fractions I and II localized at the sides of fraction III. However, he did not envision the possibility of 2 different chains covalently bound together, because all of the protein structures known at the time were composed of a single polypeptide chain. We now know Porter's I and II fractions as "Fab" (fragment antigen binding). Because fragment III could be crystallized, it was named "Fc" or crystalline fragment. The crystals of the Fc fragments coming from antibodies with different specificities were practically homogeneous. On the other hand, the lack of capacity of fractions I and II to form crystals correlated the antigenic specificity with the structural heterogeneity, in other words, differences in its aminoacid sequence. If Porter had used pepsin without a reducing agent, he would have obtained a large fragment and a great variety of smaller peptides. What he would have found in the larger fraction would have been a capacity to precipitate antigen. By adding a reducing agent, the fragment would have been divided into two fractions, as occurs with papain. Results differ, because pepsin-sensitive peptide binding is downstream from disulphide bridges joining the Fc fragment, while those sensitive to papain are upstream from the disulphide bridges. The fragments produced by pepsin are named Fab', while those that are not reduced generate a dual antigen receptor called F(ab')₂.

Rodney Porter shared the Nobel Prize for Physiology or Medicine in 1970 with Gerald M. Edelman (1929), who employed a similar strategy but with a different tactic to solve the problem of antibody structure. The first paper published on the subject also appeared in 1959.²⁰ However another one, more developed and definite, appeared in 1961.²¹ This, in contrast to Porter's, is extraordinarily elegant and delightful to read, especially the part dealing with biochemical methodology. Edelman graduated as a physician from the Universidad of Pennsylvania and his clinical training was done at the Massachusetts General Hospital. He obtained his doctorate from the Rockefeller Institute in 1960 under the tutelage of Henry Kunkel. In his 1961 paper, he collaborated with the Czechoslovakian researcher Miroslav Dave Poulik, who had immigrated to Canada, obtaining his degree as a physician in 1960 from the University of Toronto. Edelman and Poulik assumed that the antibody was composed by more than one protein. If the suspicion turned out to be right, disulphide bridges would bind the chains, as occurs with the aminoacid cysteine. Porter in fact had used cysteine, a weak reducing agent by nature, to activate papain. Edelman and Poulik chose a more potent sulphhydryl reactant, mercaptoethanol, which would break the disulphide binding if they existed, and added urea as a dissociation solvent for the resulting possible fractions. The problem of antibody heterogeneity was solved a priori thanks to the clairvoyant findings of his tutor, Henry Kunkel: employing monoclonal antibodies of patients with multiple myeloma. In fact, the following year Edelman and his student Joseph A. Gally demonstrated that Bence-Jones urinary proteins were really the low molecular weight chains of multiple myeloma,²² solving a mystery that had begun in 1845 with their discovery by the British chemist and physician Henry Bence-Jones (Figure 1).²³ But lets return to the classic study by Edelman and Poulik. Using ultracentrifuging, liquid chromatography and electrophoresis, they demonstrated that each antibody has 3 to 5 proteins. In subsequent studies the following years, Porter, Edelman and Alfred Nisonoff, among others,^{24–26} established the basic structure of IgG (Figure 2). Nisonoff used pepsin on rabbit IgG and observed the generation of a single bivalent fragment (Fab')₂ and small peptides, that is, a fragment with the capacity to bind to antigenic determinants, findings that as a whole would confirm the ideas proposed 25 years earlier by John R. Marrack¹² when he pointed out that antibodies should have at least 2 antigen binding sites. The currently accepted model of this molecule consists in 4 chains: two light (L) chains and 2 heavy (H) ones. A Y shaped configuration was proposed and then confirmed through electronic microscopy and x-ray diffraction studies. Soon thereafter 2 antigenic types of light chains, denominated κ and λ chains were described. It was observed that macroglobulin IgM was constituted by 5 structures with similarity to IgG, in a star shaped configuration where the respective Fc fragments were bound to a small polypeptide chain called the J chain. As indicated by the data of Edelman and Poulik, some disulphide bridges were not directly involved in inter-chain binding, but served to fold the linear protein and form a solid tertiary structure in which each globular domain carried out its function.

In 1965 David S. Rowe and John L. Fahey discovered a new class of antibodies, IgD, primarily restricted to immunoregulation on the B cell surface.²⁷ All of the classes of antibodies are functionally distinct and the last 2 isolated are extraordinarily specialized. The ancient immunologic riddle of local immunity against pathogenic microorganisms was resolved when Thomas B. Tomasi and his group determined that IgA (which initially had been found in the serum) could incorporate a protein component and be secreted in several fluids in the zone where the environment interacted with the host, mainly the digestive and respiratory tract. IgA is found as a 7°S monomer, as an 11°S dimer and in its 18°S secreted form.²⁸ Lastly, IgE, described in 1966 is a reagent immunoglobulin and the one responsible for the inflammatory cascade that results from the degranulation of mast cells.^{29,30}

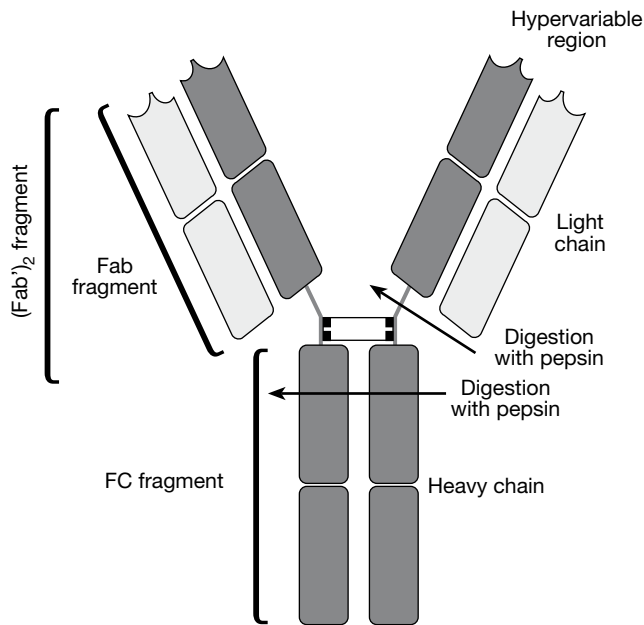


Figure 2. Monomeric unit structure of immunoglobulins. Enzymatic digestion with papain produces 2 fragments that bind the antigen (Fab) and 1 that crystallizes (Fc). Treatment with pepsin produces one large fragment (Fab)₂ and smaller ones (pepsin peptides).

Epilogue

After this panoramic vision on the history of how the chemical structure of antibodies came to be described, one could conclude that immunochemical research was complete on the subject. However, there was still a lot of information missing, although the principles had been established and, moreover, confirmed. Immunochemistry continued to have a preponderant role in the design of vaccines and diagnostic methods.

Fifty years later, we can state that antibodies are extraordinary structures, much more complex, diverse and interesting than imagined in 1959. The pioneering research by Porter and Edelman and Poulik provided information on the elemental structure of these molecules. Its fine structure, mechanisms of variability and the specificity of their receptors, the relationship of amino acid sequences with their function and other related problems occupied researchers for the rest of the century. As with the head of the hydra, the immune system responds with two questions every one that is asked.

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