ANTIBODIES: SHAPE, HOMOGENEITY, AND VALENCY

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Received 21 June 1968

The recent progress in our understanding of antibody structure and biosynthesis – while along an adventurous and tortuous path – has been simply tremendous. Two symposia volumes have appeared recently (Nobel Symposia 3, on Gamma Globulins, ed. J.Killander; and Cold Spring Harbor Symposia on Quantitative Biology, Vol. 32, on Antibodies, 1967), which bear witness to this statement. To the biochemist it is of special interest to see how closely this progress follows the prediction of Svante Arrhenius given in 1906 in his book on "Immunochemistry", namely that he will not be surprised if studies on the structure and interactions of antibodies will help, in the final analysis, the elucidation of their biosynthesis.

It is not intended to summarize here the many reports on amino acid sequence of light and heavy chains, mainly of myeloma immunoglobulins, nor to discuss the generalizations to which they led. Efforts to understand the genetic mechanisms underlying the antibody formation have resulted in a burst of theories, some of them with a half-life shorter than the period elapsed between submission to press and publication. I would like to mention here three aspects of antibody structure.

Seeing is believing!

Electron microscopy can show the general structure of immunoglobulins. This was indicated by several earlier studies, but demonstrated recently in a particularly elegant way by Valentine and Green (J. Mol. Biol. 27 (1967) 615, and the Nobel Symposium). Antibodies of the immunoglobulin G class were cross-linked by a bifunctional happen to form closed rings with various symmetrical shapes (triangles, squares, pentagons, etc.). The authors conclude that the antibody molecule is clearly very flexible for such a wide range of different shapes to be formed. It was also noticed that each corner of the various figures contained a conspicuous projection. After the preparation had been digested with pepsin, the regular shapes were still present but now without the projection. This identifies it as the Fc fragment of the molecule while the sides of the regular shapes must be the pepsin-resistant Fab fragments. They are linked at the centre of each side by the divalent hapten and at the corners by the disulphide bond which joins the two Fab fragments of each molecule. The authors found in their pictures a maximum length for the Fab fragment of 70 Å, suggesting that antibody bridges between two antigenic sites should have a maximum stretch of 140 Å.

A good glimpse has also been obtained recently of the immunoglobulin M molecule (Höglund, and Svehag et al., in the Nobel Symposium; Chesebro et al., J. Exp. Med. 127 (1968) 399). Svehag and his colleagues have shown that normal human and rabbit immunoglobulin M as well as Waldenström macroglobulins are indistinguishable from one another, when examined in the electron microscope, and reveal flexible spiderlike particles with five appendages joining a central ring. On the other hand, electron microscopy of reduced and alkylated immunoglobulin M revealed total absence of intact spider-like molecules. Thus, all the above studies show a beautiful agreement between chemical data and electron-microscopic pictures, and demonstrate

the usefulness of this tool in structural studies.

How homogeneous can antibodies be?

It has been stated again and again that an immunochemist must choose between working with heterogeneous immunoglobulins, known to combine immunospecifically with defined antigens, and working with homogeneous immunoglobulins devoid of known biological activities. Indeed, almost all the recent structural chemical studies were on myeloma immunoglobulins.

Specific antibodies are commonly very heterogeneous, and there are only a few instances in which more homogeneous populations were observed. Thus, relatively homogeneous anti-rhamnose antibodies were produced in abundance by certain New Zealand rabbits immunized with streptococci (Osterland et al., J. Exp. Med. 123 (1966) 599; Miller et al., J. Immunol. 98 (1967) 710). A restricted heterogeneity has been also reported for the light chains derived from antibodies associated with chronic cold agglutinin disease (Cohen, Nobel Symposium). These have affinity for the I antigen of the erythrocyte, and are almost always immunoglobulin M molecules with type K light chains.

Against the above background, the report of Nisonoff et al. (Cold Spring Harbor) on crystalline immunospecifically purified antibody to the *p*-azobenzoate group seems particularly dramatic. The antibody, prepared from the serum of an individual rabbit, crystallized spontaneously from cold neutral buffer solution at a concentration of 60 mg/ml. The protein redissolved at 37° and recrystallized at 5° at much lower concentrations. As expected, the antibody seemed homogeneous both in disc electrophoresis and with respect to combining affinity. The sense of drama is heightened by our being informed that the rabbit, from whose blood the antibody was isolated, is dead, and that – to our knowledge – no other rabbit has yet produced similarly crystallizable antibody.

Another approach in attempts to reduce the heterogeneity of the antibody population has been to synthesize relatively simple and well defined antigens. Haber et al. (Cold Spring Harbor) have now reported that the energy of binding the hapten by the anti-dinitrophenyl antibodies provoked in rabbit with an ordered sequence copolymer containing alanine and ε-dinitrophenyllysine, did not change with time, in contrast to the usually described increase of the binding energy as a function of time. Again, immunization of rabbits with a branched polymer in which angiotensin was bound to polylysine, led to formation of antibodies monodisperse with respect to binding affinity. Thus, an appropriate reduction in the degree of heterogeneity of antigen may modify not only the temporal rise in the association constants generally observed during the immune response, but also reduce the range of association constants present in a given antibody.

In view of the homogeneity of myeloma immunoglobulins, effectively studied only in man and in the mouse, it was natural that considerable effort should be spent on producing myeloma proteins with antibody specificity as a result of immunization. All such efforts have failed until now. On the other hand, some myeloma proteins seem to be antibodies of low avidity against definable antigens (Zettervall et al., Clin. Exp. Immunol. 1 (1966) 213). More recently, a Waldenström macroglobulin was shown to have a well-defined specificity towards the Fc portion of human immunoglobulin G (Metzger, Proc. Natl. Acad. Sci. 57 (1967) 1490; Stone and Metzger, Cold Spring Harbor).

In the same Cold Spring Harbor symposium volume M.Cohn describes one mouse myeloma which produced an antibody directed against the C-polysaccharide of the pneumococcus, whereas Eisen et al. report a human myeloma protein with anti-2,4-dinitrophenyl activity. Using a simple procedure, based on a spectral shift upon reacting ε-dinitrophenyl-L-lysine with anti-dinitrophenyl antibody, Eisen et al. screened eighty-eight sera from patients with multiple myeloma, and thus detected the anti-dinitrophenyl myeloma immunoglobulin. Its affinity for the ligand was low (2.3×10^4 M^{-1}), and its specificity resembled the low affinity anti-dinitrophenyl antibodies that appear in rabbit serum shortly after immunization is initiated.

This interesting finding raises many hopes, as we have here a molecule closest to a homogeneous antibody, but it also presents many puzzles. Is every hundredth myeloma protein an anti-dinitro-

phenyl antibody? What is the frequency of the various specificities? How specific is "specific" in this context? And finally – in the discussion of the persistence of the antigen (or hapten) in the cells producing antibodies – do we really expect to find some dinitrophenyl groups in the cells producing that particular myeloma globulin? One does not have to be a far-reaching prophet to expect many more reports about the detection of antibody activities in myeloma proteins in the near future, be they due to serendipity, clever screening schemes, or – simply – a considerable expenditure of routine labour.

Valency of macroantibodies: five or ten?

A typical immunoglobulin G antibody is divalent, i.e. it has two sites capable of combining with antigenic determinants. As immunoglobulin M (the macroglobulin) is composed of five subunits, each of a size comparable to that of immunoglobulin G (Miller and Metzger, J. Biol. Chem. 240 (1965) 3325; Lamm and Small, Biochemistry 5 (1966) 267), it might be expected that a macroglobulin antibody would have ten combining sites per molecule – if each subunit is divalent, or five combining sites – if the macroglobulin subunits are monovalent. Onoue et al. (Immunochemistry 2 (1965) 401) have found that rabbit macroantibodies against the *p*-azobenzenearsonate group bound only one *p*-iodobenzenearsonate per two heavy-light polypeptide chain pairs. Essentially no change in the number of binding sites was observed upon dissociation of the immunoglobulin M antibody into its subunits by mild reduction and alkylation. Thus, the isolated subunits seem to be univalent, notwithstanding the structural evidence which is overwhelmingly in favor of a divalent subunit.

In contrast to the above finding, Stone and Metzger (Cold Spring Harbor) observed that almost all the Fab fractions isolated from their Waldenström macroglobulin antibody were able to bind to "antigen" (in this case, human immunoglobulin G). Similarly, Merler et al. (J. Biol. Chem. 243 (1968) 386), reported that the human macroantibody to a *Salmonella typhi* O antigen had ten antigen-combining sites per molecule. Their conclusion was based on equilibrium dialysis studies of the purified macroantibodies with a tetrasaccharide isolated after partial hydrolysis from the lipopolysaccharide of *S. typhi*. The authors followed the concentration of the tetrasaccharide by absorption at 210 nm, which may be a major experimental difficulty.

Last May two additional studies on macroantibody subunits have appeared. Schrohenloher and Barry (J. Immunol. 100 (1968) 1006) investigated in the ultracentrifuge the reaction of human immunoglobulin G with subunits derived from immunoglobulin M anti-γ-globulins (rheumatoid factors), and found that the compositions of the soluble complexes formed were not altered by varying the ratio of the reactants. One immunoglobulin M subunit bound about one immunoglobulin G molecule. If the last one may be considered as a univalent antigen, the conclusion would be drawn that the macroantibody subunit is monovalent.

Frank and Humphrey (J. Exp. Med. 127 (1968) 967) have now reported a detailed study of the subunits in rabbit anti-Forssman macroantibody. The Forssman antigen is a heterogenetic antigen containing determinants, probably of mucopolysaccharide nature, common to red cells of different species, and related also to some bacterial antigens. Working at very low concentrations of antibody, Frank and Humphrey have obtained on mild reduction subunits which appear to be composed of a single heavy and light chain. Only half of these subunits could be shown to have specific binding sites for Forssman antigen. Thus, the data are consistent with the concept that each anti-Forssman immunoglobulin M molecule has five effective binding sites.

At this stage, the particular problem of macroantibody valency is in a state of healthy confusion, though undoubtedly it will be solved in the near future. From this point of view, it resembles many other problems of central interest in immunochemistry.